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PHYLOGENETIC RELATIONSHIPS WITHIN FAMILIES MYRTACEAE IN TRIPA PEAT SWAMP FOREST USING INTERNAL TRANSCRIBED SPACER (ITS)

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Abstract. The Tripa peat swamp forest is located on the west coast of Aceh province and falls administratively under the two districts of Nagan Raya and Aceh Barat Daya. Tripa peat swamp forest has a high diversity of plants. The previous research discovered that Myrtaceae is one of the dominant families in the forest. This study aimed to predict phylogenetic relationships among trees species within families Myrtaceae that live in the Tripa peat swamp forest using *Internal Transcribed Spacer* (ITS). The research was conducted at Laboratory of Forest Genetics and Molecular Forestry, Faculty of Forestry, IPB from June 2015 to December 2016. The methods were conducted with an experimental laboratory of molecular identification consisting of DNA extraction, amplification, and sequencing. Data was analyze using BioEdit, Clustal X, Mega 6, and BLAST. The morphology identification reported that there were four trees species within families Myrtaceae, i.e., *Syzygium* sp 1, *Syzygium garciniifolium*, *Syzygium leptostemon* and *Tristaniopsis whiteana*. Reconstruction of phylogenetic tree using the Neighbor-Joining (NJ) method 1000x showed that the ITS region was successfully to predict phylogenetic relationships within genus *Syzygium* and *Tristaniopsis*.

Keywords: tripa peat swamp forest, tree species, phylogenetic, myrtaceae, internal transcribed spacer

I INTRODUCTION

Tropical peat swamp forest is a unique ecosystem that is most extensive in the Southeast Asia, where it is under enormous threat from logging, fire, and land conversion. Recent research has shown this ecosystem's significance as a global carbon store, but its value for biodiversity remains poorly understood [1]. The Tripa peat swamp forest is located on the west coast of Aceh province and falls administratively under the two districts of Nagan Raya and Aceh Barat Daya. The Tripa peat swamp forest received special designation as part of the Leuser Ecosystem Zone (Kawasan Ekosistem Leuser/KEL) based on Presidential Decree No. 33/1998 on Leuser Ecosystem Management [2]. Degradation of peat swamp forest in Tripa affects the ecosystem's function such as tree species diversity and tree density. However, complete conversion of forest into a non-natural vegetation cover is a much more significant concern [2]. The impact of degradation has been a renewal of the concern over the decline of biodiversity in this forest. Ref. [3] reported there were 17 families of trees species that can be found in Tripa peat swamp forest i.e. Myrtaceae, Apocynaceae, Sapotaceae, Anacardiaceae, Sterculiaceae, Moraceae, Euphorbiaceae, Rubiaceae, Stemonuraceae, Thymelaeaceae, Ochnaceae, Rhizophoraceae, Annonaceae, Dipterocarpaceae, Myristicaceae, Elaeocarpaceae, and Arecaceae. Ref. [3] also explained that among all the families, Myrtaceae was the dominant families in the forest. Inline with Ref. [4] also reported that the most species in peat swamp forests belong to the Myrtaceae and Dipterocarpaceae working on timber species of Pondok Tanjong peat swamp forest has reported that *Syzygium grandis*, is the most dominant species recorded in two 100 m^2 study plots.

The main goal of this study is to predict phylogenetic relationships among species within families Myrtaceae. Analysis of the relationships of trees species is using *Internal Transcribed Spacer* (ITS). The nuclear *Internal Transcribed Spacer* (ITS)

regions have been used as molecular markers because of their relative variability and ease of PCR amplification [5]. The ITS consists of the entire ITS1, 5.8S and ITS2 regions of the nuclear rDNA cistron. It is a multigene family with the potential for variation among tandem repeats. ITS sequences are typically found to be more similar within species and more divergent between species [6]. Prior to, the ITS was used for phylogenetic analysis of the genus *Hexachlamys* within families Myrtaceae [7].

II METHODOLOGY

Study area

The research was conducted in Tripa peat swamp forest, Darul Makmur District, Nagan Raya (Figure 1) and Forest Genetics and Molecular Forestry Laboratory, Faculty of Forestry, Bogor Agricultural University. The research was begun from September 2015 to August 2016.

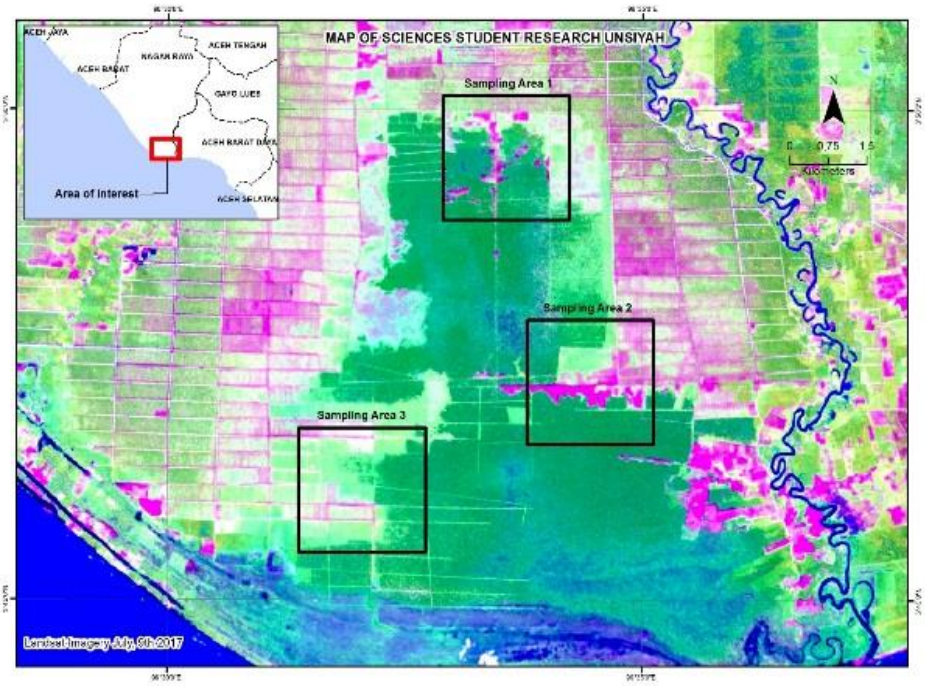

Figure 1 Location of sample collected in Tripa peat swamp forest, Aceh, Indonesia

Sample Collection

The sampling method in the field using purposive sampling and taken three different locations in Tripa peat swamp forest: primary, secondary and tertiary. The study samples were collected as leaf material from field samples. As many as fifteen trees species were collected in the field. Twelve species included in families Myrtaceae and three species included in families Arecaceae were used as an *out group*. Every leaf consisted of three replications (one sample for DNA extraction, two samples for herbaria). Samples were collected as silica gel dried leaves from natural populations and as leaves from herbaria. Voucher specimens were collected and deposited in *Indonesian Institute of Sciences* (LIPI). Plot design for collecting the samples is shown in Figure 2.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was isolated using DNeasy® Plant Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

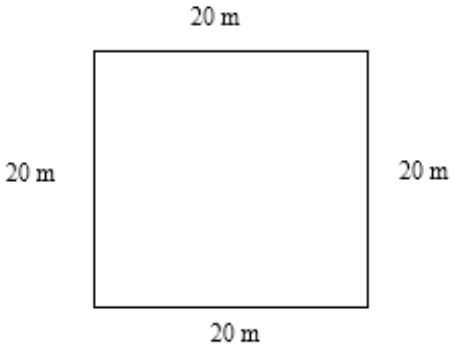

Figure 2 Sampling plot design for tree species

Amplification segments of DNA were conducted using 20 µL Polymerase Chain Reaction (PCR) reactions (Kapa *Taq* PCR MasterMix). All of the components consists of 10 µL (1X Kapa *Taq*), 1 µL forward primer, 1 µL reverse primer, 3 µL DNA template, and 5 µL nuclease-free water. The temperature for PCR condition were start to

initial denaturation at 94˚C for 3 min, 30 cycles consists of the 3 stages: denaturation (at 94˚C for 30s), annealing (at 58˚C for 30s), and extension (at 72˚C for 1 min), and ends with an elongation stage at 72°C for 10 min. The primers were used in this study in Table 1.

Table 1 Primers used in the present study

DNA region	Primer name	Sequence (5'-3')
ITS 1	ITS 1	TCCGTAGGTGAACCTGCGG
5.8S		
ITS 2	ITS 4	TCCTCCGCTTATTGATATGC

Ref. [8]

All PCR products were visualized by electrophoresis on 2% agarose gels stained with *red gel*. Amplified PCR products were sequenced based on the selective incorporation of chain-terminating dideoxynucleotides method using DNA polymerase during in vitro DNA replication according to the manufacturer's instructions and run on an ABI-3100 automatic sequencer (Applied Biosystems) [9,10]. Both DNA strands were fully sequenced.

Editing and Sequence Alignment

Data were analyzed using BioEdit, BLAST and MEGA 6. Manual data review was performed by using BioEdit *version 7.0.5.2* [11]. The *Basic Local Alignment Search Tool* (BLAST) from the NCBI homepage (http://www.ncbi.nlm.nih.gov/blast/blast.ci) was then used to compare these sequences with in-house sequences and GenBank database sequences. The software of *Molecular Evolutionary Genetics Analysis* (MEGA) *version 6.0* [12] was used to predict phylogenetic relationships among trees species in Tripa peat swamp forest. The reconstructing of the phylogenetic tree using Neighbor Joining method (NJ) bootstrap 1000x [13].

III RESULT AND DISCUSSION

The morphological identifications showed that there were four trees species from families Myrtaceae, i.e. *Syzygium* sp., *Syzygium garciniifolium*, *Syzygium leptostemon*, and *Tristaniopsis whiteana*. More details about all species are presented in Table 2. The most dominant species of the tree in Tripa peat swamp forest was *Tristaniopsis whiteana*. This species was discovered almost in every location included three areas, i.e. primary, secondary, and tertiary. The morphology characters of *T. whiteana* that were discovered in Tripa peat swamp forest, i.e. tree with height>20 meters, the diameter of tree around 20-30 cm, terms of its peeling barks, has small flowers, and small leaves.

Table 2 The species of trees that exist in Tripa peat swamp forest, all Familes belong to Myrtaceae.

Species name	Genus	Total of individuals
Syzygium sp.	*Syzygium*	5
Syzygium garciniifolium	*Syzygium*	41
Syzygium leptostemon	*Syzygium*	6
Tristaniopsis whiteana	*Tristaniopsis*	199

Inline with [14] was reported that *Tristaniopsis* is distinctive in terms of its peeling barks with mixed reddish brown to gray-white. According to Ref. [6] morphological characteristics of *T. whiteana*, i.e. has a canopy, occasionally shortly emergent tree to 45 m high, to 1.5 m with concavely rounded buttresses. Bark at first was white to light greenish grey with the occasional hint of very pale orange, smooth, later peeling in scroll-like strips; the older unpeeled bark was evenly pale grey-dull light olive; peeled bark scrolls were pale to dark grey with mauve-brown patches; the inner bark was whitish. The species of *T. whiteana* at Tripa peat swamp forest is shown in Figure 3.

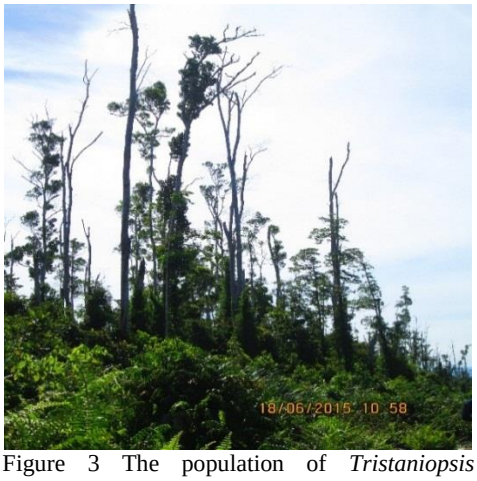

Figure 3 The population of *Tristaniopsis whiteana* in Tripa peat swamp forest

Distribution of *Tristaniopsis whiteana* in Tripa peat swamp forest was not only in low peat-thickness but also in high peat-thickness. Based on the distribution, it can be assumed that *T. whiteana* favorably grew in acid soils. According to Ref. [4], genus *Tristaniopsis* is one of the tree species that grow in peat forest ecosystem. In the world, distribution of *Tristaniopsis*

whiteana is in Sumatra, Peninsular Malaysia (including Singapore) and Borneo. More abundant were found in Sarawak and SW Sabah than elsewhere in Sabah [15]. Generally, *T. whiteana* is classified as timber forest products. The local names of *T. whiteana* are pelawan, belawan putih, malu tua, etc.

Table 3 BLAST analysis of 4 sequences sample based on ITS region

	Process-id	Quary Cover	Ident	E-value
Syzygium sp. 1	>KC815990.1 *S. samarangense*	100%	92%	0.0
	>KM064931.1 *S. australe*	94%	92%	0.0
	>FJ972836.1 *Syzygium sp. 1*	98%	91%	0.0
	>KM064818.1 *S. floribundum*	93%	91%	0.0
	>KM064864.1 *S. smithii*	93%	91%	0.0
Syzygium garciniifolium	>KC815990.1 *S. samarangense*	100%	97%	0.0
	>FJ972836.1 *Syzygium sp. 1*	98%	96%	0.0
	>FJ972837.1 *Syzygium sp. 2*	98%	95%	0.0
	>KM064993.1 *S. paniculatum*	93%	95%	0.0
	>AM234135.1 *S. jambos*	93%	94%	0.0
Syzygium leptostemon	>KC815990.1 *S. samarangense*	100%	98%	0.0
	>FJ972836.1 *Syzygium* sp. 1	99%	96%	0.0
	>KC815991.1 *S. jambos*	100%	96%	0.0
	>KP093045.1 *S. buxifolium*	90%	95%	0.0
	>KP093048.1 *S. hancei*	90%	95%	0.0
Tristaniopsis whiteana	>EF041514.1 *T. laurina*	94%	98%	0.0
	>KM064886.1 *T. laurina*	92%	98%	0.0
	>KM064872.1 *T. laurina*	91%	98%	0.0
	>KM064824.1 *T. laurina*	91%	98%	0.0

Basic Local Allignment Search Tool (BLAST)

The BLAST result analysis of 4 trees species within families Myrtaceae from Tripa peat swamp forest is presented in Table 3. The table shows that all species of trees within families Myrtaceae from Tripa peat swamp porest had a high level of *similarity* with the sequences in GenBank DNA database at the genus levels. The *Query Cover* for four trees species has a values in the range of 91-100%. A *Query Cover* is a number that describes how much of the query sequence is covered by the target sequence. If the target sequence in the database spans the whole query sequence, then the query cover is 100%. The *E-value* for all species of trees is 0.0. Based on *E-value*=0.0, we can assume that the sequence is identical to the BLAST sequence result. The lower the *E-value* is, the more significant the match. According to Ref [16] explained the *E-value* in BLAST takes into account both the length and composition of the alignment along with the percentage identity found. A number close to 0.0 means that the hit has to be significant.

Phylogenetic relationships Based on *Internal Transcribed Spacer* (ITS) region

The phylogenetic tree in Figure 4. explained about phylogenetic relationships among species within families Myrtaceae. Based on the result, ITS region was successful in grouping the species originated from Tripa peat swamp forest (TPSF) with species from GenBank (GB) into the same *clade*. It can be presumed that *Syzygium* sp. 1 (TPSF) together with *Syzygium australe* and *Syzygium samarangense* were from a single common ancestor. Inline with *Syzygium garciniifolium* (TPSF), this species also formed the *monophyletic group* together with four species from GenBank. According to [17] *clade* is a piece of a phylogeny that includes an ancestral lineage and all the descendants of that ancestor. This group of organisms has the property of monophyly, so it may also be referred to as a *monophyletic group*.

Based on morphological *Syzygium* sp. 1 and *S. australe* are two different species. *Syzygium* sp. 1 is species originated growing in peat swamp forest. On the other hand, *S. australe* is a common plant of coastal and highland rainforests from Southern New South Wales to central Queensland. In nature, it can reach over 25 m in height, but it is usually smaller in cultivation. The leaves are opposite (each pair emerges from the stem at the same location but on opposing sides), rounded and bright, glossy green in color [18]. Conversely, refer to-molecular identifications *S. australe* is *suggested* as the *sister group* to *Syzygium* sp. 1 for the future studies. In addition, *S. samarangense* also recommended as the *sister groups* to *S. garciniifolium*, together their formed the same branches with 66% bootstrap support. According to Ref. [19] *sister group* is a phylogenetic term denoting the closest relatives of another given unit in an evolutionary tree.

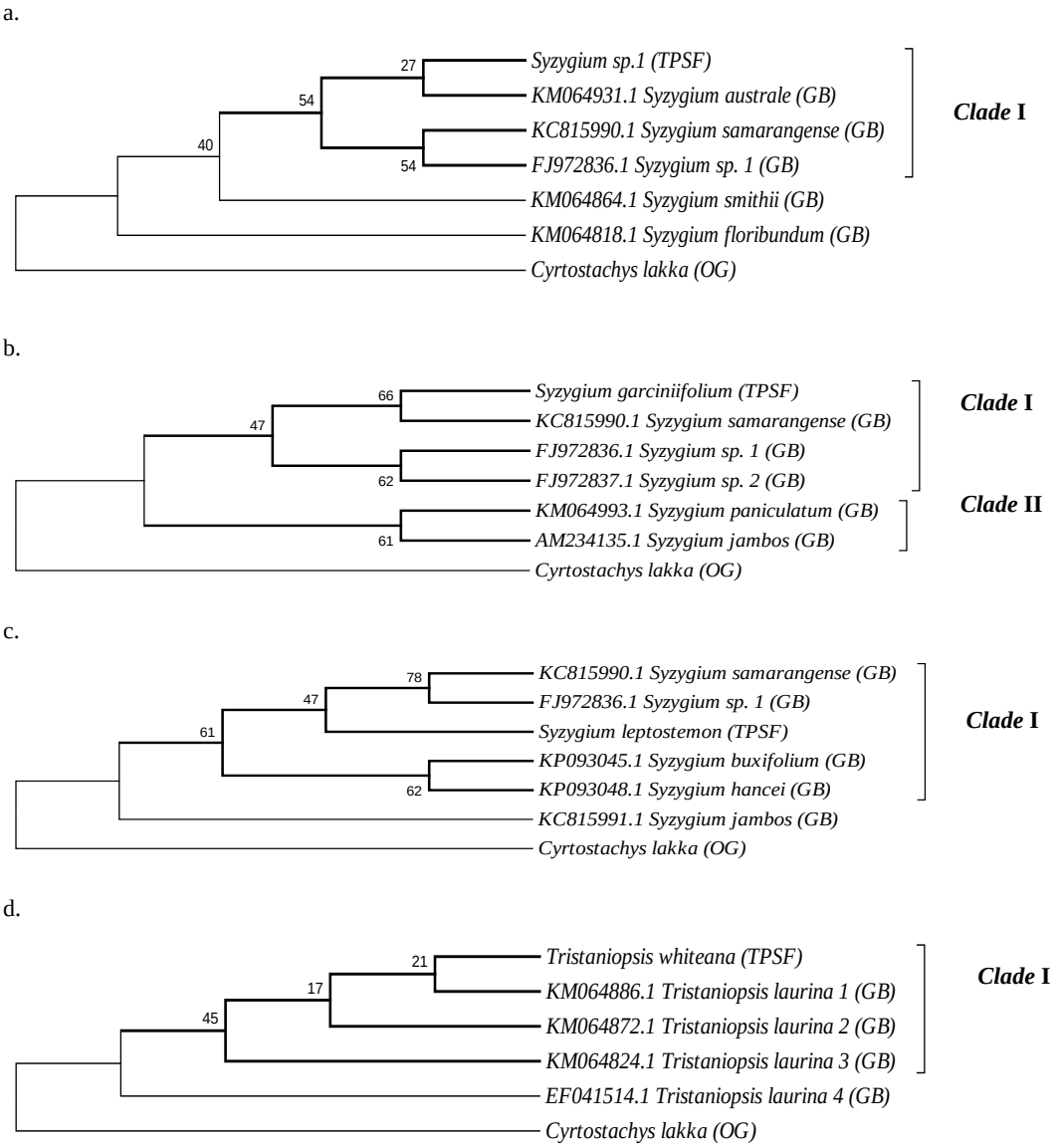

Figure 4 Phylogenetic tree based on *Internal Transcribed Spacer* (ITS) region within spesies: (a) *Syzygium* sp.1 (b) *Syzygium garciniifolium* (c) *Syzygium leptostemon* and (d) *Tristaniopsis whiteana* using Neighbor-Joining (NJ) method 1000x (TPSF : Tripa Peat Swamp Forest, GB : GenBank, OG : *Out Group)*

Different case to *Syzygium leptostemon*, this species has two of the *sister group*, i.e. *S. samarangense* and *Syzygium* sp.1. Although *S. leptostemon*, *S. samarangense*, and *Syzygium* sp.1 include in genus *Syzygium*, but based on morphological characteristics they are different species. *S. samarangense* is the native species grown throughout the Southeast Asian countries, such as, Indonesia, Malaysia, Thailand and Taiwan [20]. This species in Indonesia is known as jambu air. Otherwise, *S. leptostemon* is one of the species as the compiler of peat swamp vegetation in Tripa peat swamp forest. According to Ref. [21] reported that *S. leptostemon* was discovered in peat swamp of Danau Sentarum National Park Kapuas Hulu Regency. The previous research reported that the *Internal Transcribed Spacer* (ITS) has been used to predict of representatives in genus *Syzygium* within families Myrtaceae [5].

Phylogenetic relationships within genus *Tristaniopsis*, showed that *Tristaniopsis whiteana* (TPSF) and *Tristaniopsis laurina* (GB) are closely related. Based on the distribution of species, *T. laurina* is a native species in Australia. It usually grows near the eastern coastline and along the banks of streams. Whereas, *T. whiteana* is a native species in Sumatra, Peninsular Malaysia, Singapore, and Borneo [15]. Overall, no one tree species from Tripa peat swamp forest formed phylogenetic relationships with the

same species. Due to DNA GenBank database does not provide the same sequence for each species. In the future, it is very important to develop of studies about forest genetic in the tropical rainforest, especially species of tree from peat swamp forest. Here, *Internal Transcribed Spacer* (ITS) is recommended as candidate barcode for the tree species from peat swamp forest. According to Ref. [22] among six tested loci i.e. *mat*K, *rbc*L, *rpo*B, *rpo*C1, *trnH-psbA*, and ITS. The ITS is recommended as a possible *barcode* for plants, providing 100% species identification. In line with Ref. [23], the *Internal Transcribed Spacer* (ITS) of nuclear ribosomal DNA is one of the most commonly used DNA markers in plant phylogenetic and DNA barcoding analyses and it has been recommended as a core plant DNA barcode.

CONCLUSION

Internal Transcribed Spacer (ITS) region was successful to predict the phylogenetic relationships among four species within families Myrtaceae. Also, the ITS region is recommended as a candidate barcode for the tree species from peat swamp forest.

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REFERENCE

1. Mary RCP, Lahiru SW and Richard TC 2011 Biodiversity and Conservation of Tropical Peat Swamp Forests. *Bioscience* **61** (1):49.
2. Widayati A, Tata HL, Rahayu S and Said Z 2012 Conversions of Tripa peatswamp forest and the consequences on the loss of Sumatran Orangutan (Pongo abelii) habitat and on aboveground CO2 emissions. Brief No. 33: Tripa series. Bogor, Indonesia. World Agroforestry Centre (ICRAF), Southeast Asia Regional Program.
3. Thomy Z, Yasmin Y and Masykur 2016 Keanekaragaman Jenis Tumbuhan Pada Hutan Rawa Gambut Tripa, Aceh. Seminar Nasional Biodiversitas. UNS. Solo.
4. Handayani D, Achmadi SS and Agusta A 2014 Antibacterial Compounds of Belawan Putih (Tristaniopsis whiteana) Leaves. *J. Biologi Indonesia* **10**(1): 93-100.
5. Biffin E, Harrington MG, Crisp MD, Craven LA and Gadek PA 2007 Structural partitioning, paired-sites models and evolution of the ITS transcript in Syzygium and Myrtaceae. *Molecular Phylogenetics and Evolution* **43**(1):124-139.
6. Alvarez I and Wendel JF 2003 Ribosomal ITS sequences and plant phylogenetic inference. *Mol. Phylogenet Evol.* **29**:417–434.
7. Fernanda DA, Cruz, Andreia C, Turchetto-Zolet, Nicole V, Claudio A.M, Marcos S, Mauricio A and Rogerio M 2013 Phylogenetic analysis of the genus Hexachlamys (Myrtaceae) based on plastid and nuclear DNA sequences and their taxonomic implications. *Botanical J. of the Linnean Society*. **172**:532–543.
8. White TJ, Bruns T, Lee S and Taylor J 1990 *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications.* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, New York, USA. 315–322.
9. Sanger F and Coulson AR 1975 A Rapid Method for Determining Sequences in DNA by Primed Synthesis with DNA polymerase. *J. Mol. Biol.* **94** (3): 441–8.
10. Sanger F, Nicklen S and Coulson AR 1977 DNA Sequencing with Chain-terminating Inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74** (12): 5463–7.
11. Hall TA 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. **41**:95–98.
12. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S 2013 Molecular Evolutionary Genetics Analysis version 6.0. *Molecular J. Biology and Evolution.* **30**(12):2725-2729.
13. Saitou N and Nei M 1987 The Neighbor Joining Method: a New Method for Reconstruction Phylogenetic Trees. *Molecular Biology and Evoution.* **4**: 406-425.
14. Öztürk M, Hakeem KR, Hanum F and Efe R 2015 *Climate Change Impacts on High*

Altitude-Ecosystems. Springer Cham Heidelberg New York Dordrecht, London.
15. Ashton PS 2005 New Tristaniopsis Peter G.Wilson & J.T.Waterh. (Myrtaceae) From Borneo. *Gardens' Bulletin Singapore*. **57**:269-278.
16. Claverie J and Notredame C 2003 *Bioinformatics for Dummies*. Indianapolis (US): Wiley Publishing.
17. Baum D 2008 Reading a Phylogenetic Tree: The Meaning of Monophyletic Groups. *Nature Education*. **1**(1):190.
18. Australian Native Plants Society (Australia) (ANPSA). Syzygium australe. http://conference2015.anpsa.org.au/saus.html. (accessed on March 1, 2018).
19. Eernisse DJ 2012 *Introduction to Phylogeny: What is a Sister Taxon?*.Biology 404-Evolution. Department of Biological Science, California State University, Fullerton.
20. Khandaker MM, Boyce AN, Osman N and Sharif Hossain ABM 2012 Physiochemical and Phytochemical Properties of Wax Apple (Syzygium samarangense [Blume] Merrill & L. M. Perry var. Jambu Madu) as Affected by Growth Regulator Application. *J. The Scientific World*. **13**:1-2.
21. Randi A, Manurung TF and Siahaan S 2014 Identification of Tree Species as the Compiler of Peat Swamp Vegetation in Danau Sentarum National Park Kapuas Hulu Regency. *J. Hutan Lestari*. **2**(1):71-72.
22. Singh HK, Parveen I, Raghuvanshi S and Babbar SB 2015 The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of Dendrobium species. *J. BMC Research.* **5**(42):2-3.
23. Cheng T, Xu C, Lei L, Li C, Zhang Y and Zhou S 2016 Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. *Mol Ecol Resour*. **16**(1):138-49.